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Hepatitis C and Human Immunodeficiency Virus RNA Degradation by Methylene Blue/Light Treatment of Human Plasma

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Treatment of human plasma with methylene blue in combination with visible light (MB/light) inactivates several bloodborne viruses such as retro viruses and herpes viruses. The viral nucleic acid is thought to be a critical target for the inactivation procedure. We investigated the effects of photodynamic treatment on the RNA of hepatitis C virus (HCV) and human immunodeficiency virus type 1 (HIV-1) using Amplicor reverse transcriptase polymerase chain reaction (RT-PCR), which detects and quantifies a small fragment of the viral RNA.

The detectable HCV RNA load (5-nontranslated region) in infected human plasma declined by 94–97 % within 10 min of illumination in small-scale experiments (1–2 ml vol.). Since the same effect was observed in both anti-HCV positive and negative (''window'') samples, it can be concluded that HCV antibodies do not influence virus inactivation by photodynamic treatment.

The effect of treatment on RT-PCR signals of HIV-1, which is known to be inactivated rapidly by MB/light treatment, was examined. Plasma was infected with HIV-1 and subjected to RT-PCR, which detected a part of the *gag* gene. The extent and kinetics of PCR signal reduction induced by MB/light treatment were similar to those observed for HCV.

Experiments at production scale where single plasma units (300 ml) were infected with HCV showed reduction rates of PCR signals consistent with those measured in the small-scale experiments.

The data support the view that MB/light treatment affects the viral nucleic acids and suggest that HCV is susceptible to the procedure. ***J. Med. Virol. 56:239–245, 1998.***

KEY WORDS: virus inactivation; photosensitization; RT-PCR;RNA damage; methylene blue; HCV

INTRODUCTION

HCV, HIV, and cytomegalovirus are the major causes of blood transfusion-transmitted diseases [Sloand et al., 1995; Sloand, 1997]. An important limitation of current serological screening for HIV and HCV is the failure of tests to detect blood donors who are in the "window" phase of infection. The risk of transmitting infections from such donors can be reduced further by methylene blue treatment of human plasma [Lambrecht et al., 1991]. Under the production conditions of MB/light-treated single plasma units, it was shown that several viruses such as retro viruses and herpes viruses are inactivated [Mohr et al., 1993; Lambrecht et al., 1994]. Hog Cholera and West Nile viruses are also inactivated, suggesting susceptibility of the closely related HCV to MB/light treatment [Mohr et al., 1995].

As HCV cannot be assayed in tissue cultures, its susceptibility to inactivation procedures must be measured by other methods. Since the viral nucleic acid is thought to be a critical target structure for virus inactivation by MB/light [Wallis and Melnick, 1965; Abe and Wagner, 1995; Müller-Breitkreutz and Mohr, 1997], the measurement of nucleic acid damages may be a useful tool. The photoactivity of MB toward isolated nucleic acids has been examined for both DNA and RNA, showing a broad range of damage [Schneider et al., 1990, 1993; Epe et al., 1993; Kvam et al., 1994].

For HIV-1, RNA damage induced by MB/light treatment has been demonstrated by a loss of the PCR signal using a qualitative RT-PCR assay [Bachmann et al., 1995]. A similar effect is observed with MB/light-treated HCV [Mohr et al., 1997]. PCR assays are also used to monitor virus damage in other inactivation procedures, e.g., psoralen/UVA [Lin et al., 1993] or heat

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treatment [Hart et al., 1992, 1993]. The extent and the kinetics of the effects remained unclear in these studies because of the qualitative nature of the tests. Nevertheless, the reduction of HIV-1 infectivity upon exposure to heat was apparently more rapid than the loss of the PCR signal [Hart et al., 1993]. This is not surprising considering that in PCR assays only a small fragment of the viral nucleic acid is monitored, whereas other genomic regions and virus structures are also likely to be damaged, which can contribute to virus kill. However, PCR results may give a better understanding of virucidal procedures and useful additional knowledge of the susceptibility of viruses that cannot be assayed in tissue culture.

The aim of this study was to quantify the effects of MB/light on the integrity of RNA in HCV and HIV-1. The kinetics of photoinduced damage were measured using quantitative PCR technology. The influence of virus antibodies on the effectivity of treatment was examined and the attack on HCV RNA was monitored under production conditions for MB/light-treated plasma.

MATERIALS AND METHODS

Human Plasma and Chemicals

Human fresh plasma was obtained from blood by centrifugation (4,000 ×g, 10 min) and stored at – 30°C. Culture media and sera were obtained from Gibco BRL (Eggenstein, Germany). Methylene blue was from Merck (Darmstadt, Germany). RT-PCR kits were purchased from Hoffmann-La Roche (Grenzach, Germany).

Viruses

Anti-HCV-positive plasma was obtained from infected blood donors. An HCV-seroconversion panel from Bioclinical Partners (Franklin, MA) was used for experiments with anti-HCV-negative, HCV RNA-positive samples. Seronegative panel members were pooled and diluted in fresh plasma. HIV-1 (HTLV-IIIB) was propagated in Molt4 cells. Infected cells were grown at 37°C in 5 % CO_2. RPMI 1640 media were supplemented with 10 % fetal calf serum, 0.4 mg/ml glutamine, 0.1 mg/ml streptomycin and 0.1 mg/ml neomycin. To harvest the viruses, contaminating cells were removed by low-speed centrifugation (700 xg, 10 min and 10,000 xg, 15 min). Virus was then concentrated at 40,000 xg for 60 min and used to spike human plasma. Final plasma concentration was > 99.999 %.

MB/Light Treatment

For photodynamic treatment of viruses in small-scale experiments, 1–2 ml plasma samples in polycarbonate round bottom tubes (Falcon) were used. When the production procedure for MB/light-treated fresh plasma was used, single units of fresh frozen plasma in plastic bags from Baxter were spiked with virus (final volume: 300 ml); 1 μM MB (final concentration) was added to the samples, which were then incubated in the dark for 1 h at 4°C and illuminated with fluorescent tubes at room temperature as described elsewhere [Mohr et al., 1992]. Fluence rate was 50,000 Lux in small-volume experiments and 45,000 Lux under production conditions, as measured using an Illuminance Meter T-1M (Minolta, Ahrensburg, Germany). At different time intervals during illumination, samples were withdrawn and analysed immediately. Control samples were illuminated without MB or incubated in the dark (at room temperature). All examinations were carried out in three independent experiments.

RT-PCR Assays

Amplicor Monitor™ assays (Hoffmann-La Roche) were used for quantitative PCR analysis of HCV- and HIV-1-infected samples. In the HCV assay, the primers KY80 and KY78 are used. Target RNA is a fragment of 244 bases of the 5-nontranslated region (5-NTR) of HCV [Young et al., 1993]. In the HIV-1 assay, primers SK431 and SK462 are used to amplify a fragment of 124 base pairs within the *gag* gene [Mulder et al., 1994]. The assays quantify RNA by calibration with an internal RNA standard processed in each sample. The procedure was performed according to the protocol of the manufacturer. Samples with PCR results > 4×10^5 copies/ml for HCV or > 7×10^5 copies/ml for HIV-1 were diluted in human fresh plasma. Each sample was measured in duplicate or at different dilutions.

RESULTS

Kinetics of HCV RNA Damage in Small-scale Experiments

To gain a better understanding of HCV RNA susceptibility to MB/light photosensitization, treated samples were analysed by quantitative RT-PCR for detection of 5-NTR of HCV. For photodynamic treatment of viruses in small-scale experiments, 2 ml anti-HCV-positive plasma in polycarbonate round bottom tubes were used. The initial concentration of HCV RNA was 1 – 1.5 × 10^6 copies/ml. Samples were incubated with 1 μM MB in the dark and then illuminated at 50,000 Lux.

The linearity of the PCR results and the development of PCR inhibitors following MB/light treatment of the plasma were checked by diluting fresh plasma in MB/light treated or untreated plasma followed by RT-PCR. In both cases, the signals showed linearity with the dilution step (Figs. 1 and 2). The linear range of the assay was ~ 4×10^3 – 4×10^5 copies/ml. In some experiments, elevated PCR signals in samples with lower RNA load were observed (data not shown). Treatment of samples with MB/light resulted in a significant reduction of the PCR signal (Fig. 2). When viruses were diluted in MB/light-treated plasma (containing 1 μM MB, illumination for 30 min with 50,000 Lux) or in untreated plasma, no difference was observed in PCR results for target RNA or for the internal RNA standard (data not shown). Thus the treated plasma did not interfere with the test results by inhibiting the PCR.

Virus containing samples were treated with MB/light using different illumination times. The RT-PCR signals were measured in duplicate and mean values

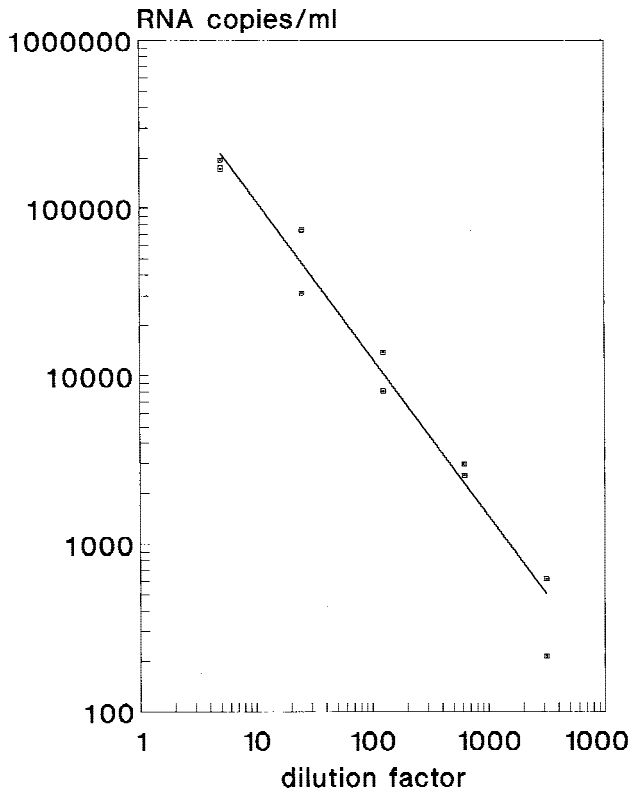

Fig. 1. Quantitative RT-PCR with HCV-infected plasma. Infected plasma was diluted with fresh human plasma and subjected to quantitative Amplicor RT-PCR. Two measurements are shown at different dilution steps. The straight line is a least-square fit.

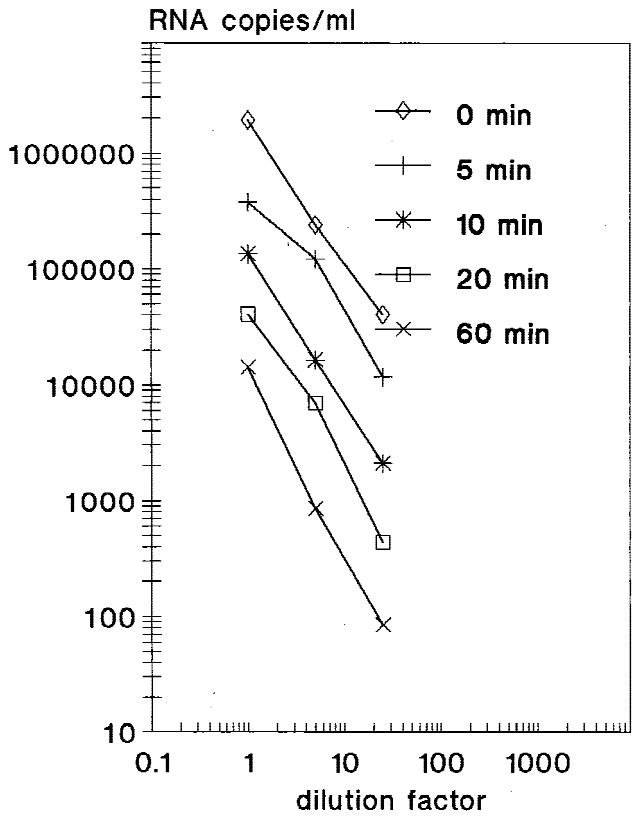

Fig. 2. Linearity of PCR results obtained with MB/light-treated HCV-infected plasma samples (2 ml vol., anti-HCV positive), which were treated with MB/light. At different times of illumination (50,000 Lux), samples were subjected to quantitative RT-PCR in different dilution steps.

were calculated. To demonstrate the effect of illumination on the MB-containing samples, the PCR signals were expressed as a percentage of those signals found in the virus-containing control samples before illumination, i.e., after preincubation (Fig. 3). In each of three independent experiments, an illumination of 10 min resulted in a reduction of the PCR signal by 94–97 %. The effect of illumination on the RNA decreased with increasing illumination time. After 60 min of illumination, the initial signal was reduced by > 98.5 % (Fig. 3). MB alone or illumination in the absence of dye had no significant influence on the PCR signals.

No Influence of HCV Antibodies on Sensitivity of HCV

In the experiments described above, plasma containing HCV as well as antibodies to the virus was used. In the following experiments, "window" plasma, i.e., anti-HCV negative but HCV RNA-positive, was treated with MB/light. Sample volume was 1 ml. The PCR signals were reduced by 91–97 % in the first 10 min of illumination (Fig. 4). Compared with the results obtained with anti-HCV-positive samples (Fig. 3), HCV antibodies did not seem to influence the susceptibility of HCV to photodynamic damage.

Kinetics of HIV-1 RNA Damage

HIV-1 is known to be highly sensitive to MB/light treatment as measured by infectivity assays [Lambrecht et al., 1994]. Furthermore, qualitative RT-PCR results become negative after illumination of HIV-1 in the presence of MB [Bachmann et al., 1995]. Attempts were made to quantify this effect by infecting human plasma with HIV-1 and treating it with MB/light in small-scale experiments. The initial concentration of RNA was adjusted to ~0.6×10^6 copies/ml. An illumination of 10 min resulted in a reduction of the PCR signals by 88–90 %. As also observed with HCV, the effect of illumination on HIV-1 RNA decreased with increasing time. After 20 min, 97–98 % of the initial signal was destroyed. After 60 min of illumination, the signal was reduced by > 98.5 % (Fig. 5).

The kinetics of signal reduction for HCV and HIV-1 were similar, with HIV-1 apparently slightly more resistant to MB/light treatment: After 10 min of illumination, 91–97 % of the HCV RNA signals were destroyed, compared to 88–90 % measured with HIV-1 (Figs. 4 and 5).

HCV RNA Damage Under Production Conditions

The production procedure for MB/light-treated plasma for therapeutic use was simulated by treating single units of fresh frozen plasma. Anti-HCV-positive plasma was added to the plastic bags to obtain a con-

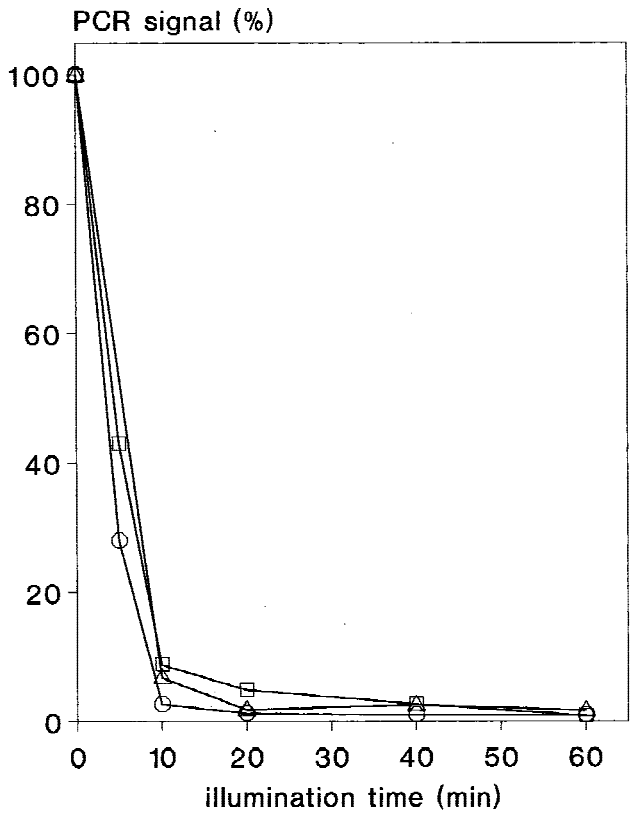

Fig. 3. PCR analysis of HCV RNA during MB/light treatment of anti-HCV-positive plasma. PCR signals are expressed as the percentage of the sample measured at 0 min of illumination (mean values of two measurments were used). Results of three independent small-scale experiments are shown.

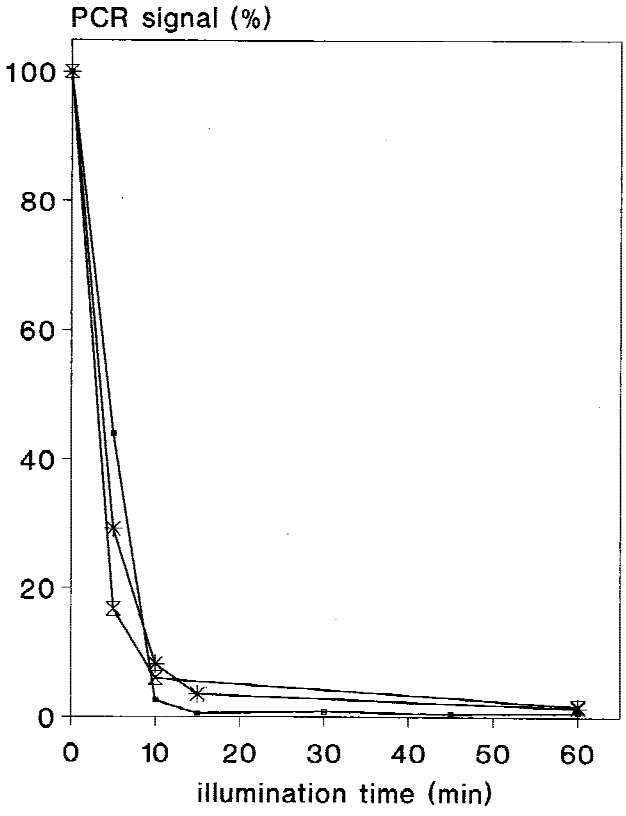

Fig. 4. PCR analysis of HCV RNA during M/light treatment of anti-HCV-negative plasma ("window" plasma). PCR signals are expressed as the percentage of the sample measured at 0 min of illumination (mean values of two measurments were used). Results of three independent small-scale experiments are shown.

centration of ~1.5 × 10^6 RNA-copies/ml. Illumination was carried out for 60 min at 45,000 Lux (this is the lowest fluence rate permitted for the production of MB/light-treated plasma). After 10 and 60 min of illumination, PCR signals were reduced by 91–97 % and > 98.3 %, respectively (Fig. 6). These findings are consistent with the results of the small-scale experiments.

DISCUSSION

The goal of the present study was to determine the effects of MB/light treatment on the RNA of HCV and HIV-1 in human plasma. The effect on HCV was of special interest because HCV is the major causative agent for posttransfusion hepatitis [Heintges and Wands, 1997; Sloand, 1997], and its susceptibility to inactivation procedures cannot be assayed by tissue culture.

The amount of detectable 5-NTR of HCV was decreased to a large extent (91–97 %) within 10 min of illumination in the presence of 1 μM MB. When HIV-1-infected plasma was examined by RT-PCR, the effects were similar to those found for HCV. These findings are consistent with the view that the viral nucleic acid is damaged by MB/light treatment. It is assumed that MB penetrates the virus coat and binds to its nucleic acid, which is thought to be a main target structure of the photodynamic action [Wallis and Melnick, 1965; Abe and Wagner, 1995]. The viral envelopes and capsids do not appear desintegrated when examined by electron microscopy after MB/light treatment [Müller-Breitkreutz and Mohr, 1997]. Inactivated herpes viruses are not impaired in their ability to adsorb to host cells or to release their DNA within the cells [Schnipper et al., 1980; Müller-Breitkreutz and Mohr, 1997].

Since the inactivation procedure does not seem to destroy the integrity of the viruses or to free viral nucleic acids, the declining RT-PCR signals described in the present study are supposed to reflect a direct effect of treatment on the viral RNA rather than indirect effects such as an enhanced sensitivity of treated viruses to enzymatic degradation. Other virus inactivation methods such as pasteurisation or solvent detergent treatment are known to destroy the infectivity of several RNA or DNA containing viruses, but do not significantly affect their detectability by PCR [Hilfenhaus et al., 1997]. In contrast to this observation, a few minutes of illumination in the presence of MB were sufficient to reduce strongly the detectability of viral RNA. These results presumably reflect the differences in the virucidal mechanisms of the procedures with MB/light acting more specifically on the nucleic acids.

Lin et al. [1993] developed a "PCR inhibition assay"

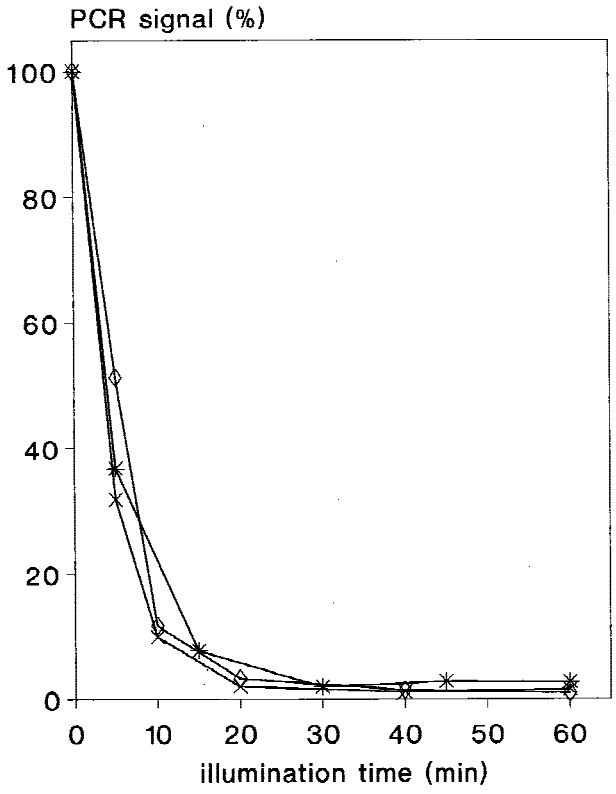

Fig. 5. PCR analysis of HIV-1 RNA during M/light treatment of HIV-1-infected plasma. PCR signals are expressed as the percentage of the sample measured at 0 min of illumination (mean values of two measurments were used). Results of three independent small-scale experiments are shown.

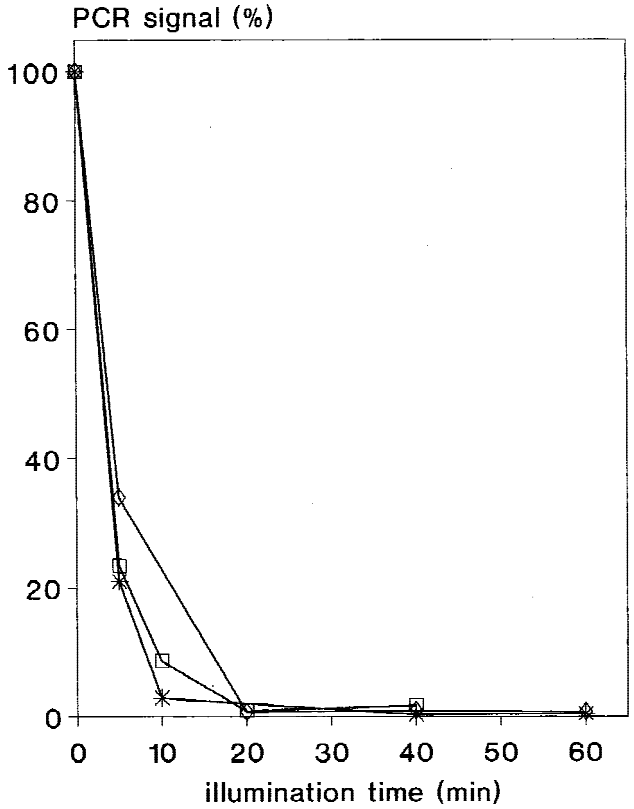

Fig. 6. PCR analysis of HCV RNA during MB/light treatment of HCV-infected plasma under production conditions. Single units of fresh frozen plasma (300 ml vol) were spiked with anti-HCV-positive plasma and treated with MB/light. PCR signals are expressed as the percentage of the sample measured at 0 min of illumination (mean values of two measurments were used). Results of three independent experiments are shown.

to estimate the efficiency of inactivation of proviral nucleic acid sequences by psoralen/UVA-treatment. The PCR signal reduction corresponded with the psoralen-DNA-adduct formation during photodynamic treatment of cell-associated HIV-1 [Lin et al., 1993]. In the case of RT-PCR analysis of MB/light-treated samples, the reason for the loss of PCR signals must be different. MB exhibits affinity for binding to isolated DNA [OhUigin et al., 1987; Fujimoto et al., 1994; Tuite and Norden, 1994] and RNA [Antony et al., 1993], but does not covalently link to nucleic acids [Tuite and Kelly, 1993]. Illumination results in strand breaks, base modification, loss of bases [Schneider et al., 1990,1993; Epe et al., 1993; Kvam et al., 1994) and crosslinks of RNA to proteins [Singh and Ewing, 1978]. Guanine residues appear particularly sensitive and are involved in most cases of damage. They also seem to play a critical role in the sensitivity of viruses to MB/light treatment [Thiry, 1966]. In the RT-PCR assays with MB/light-treated HCV and HIV-1, they probably caused the reduction of PCR signals by inhibiting primer annealing and/or extension.

The virucidal effects of MB/light photosensitization are clearly demonstrated for HIV-1 and several other viruses using tissue culture infectivity assays [Lambrecht et al., 1991; Mohr et al., 1993,1997]. An illumination of 10 min is sufficient to inactivate > 6.3 $\log_{10}$ steps HIV-1 assayed in human lymphoma MT-4-cells [Lambrecht et al., 1994]. In the present study, the estimate of PCR signal reduction was ~1 log10 step (88–90 %) during the same interval of illumination. Thus the effect of photosensitisation on PCR signals was much smaller than on infectivity. This finding is not unexpected: PCR assays monitor exclusively a small fragment of the viral nucleic acid (in the present study a fragment within the *gag* gene), but other genomic regions are likely to be damaged as well and this most probably contributes to virus kill. Furthermore, other virus structures such as proteins can also be targeted [Bachmann et al., 1995].

The extent and the kinetics of PCR signal reduction during MB/light treatment of HCV were quite similar to those of HIV-1, with HCV being slightly more sensitive. The amount of detectable 5-NTR of HCV (location of the target RNA fragment) was decreased by 91–97 % within 10 min of illumination in small-scale experiments and under production conditions of single plasma units. Although 5-NTR of HCV is known to be essential for virus replication (it carries an internal ribosome entry site) [Brown et al., 1992; Tsukiyama-Kohara, 1992], the full implications of the RNA damage observed for the infectivity of HCV are still un-

clear. However, the results obtained for HIV-1 where the PCR method underestimated the true effect of the procedure on viral infectivity suggest that the effect on HCV infectivity is presumably greater than the measured reduction rates of PCR signals. The virucidal effects of the MB/light procedure, which were clearly demonstrated for HCV model viruses such as Semikli Forest, West Nile, or Hog Cholera virus, support this assessment. These viruses are inactivated by >6 $\log_{10}$ steps during MB/light treatment [Mohr et al., 1992, 1995].

In conclusion, the reported RT-PCR results, together with the proven inactivation of related viruses, suggest a good susceptibility of HCV to the photodynamic procedure. To evaluate further the RT-PCR assay as a surrogate test for the sensitivity of HCV to MB/light treatment, it would be important to use a model virus such as Hog Cholera virus for a comparative analysis of 5-NTR by RT-PCR and infectivity by tissue culture assay.

The MB/light procedure described here has been used since February 1992 at the Blood Center of the German Red Cross Chapters of NSOB, Institute Springe, for routine treatment of single donor units of fresh frozen plasma [Mohr et al., 1993]. The benefits of virus inactivation procedures for the safety of plasma products are, apart from the inactivation of newly emerging and yet unknown viruses, the inactivation of infective units that escape routine detection because of a lack of antibodies in donors who are in the "window" phase of infection or because of GLP failures in the screening laboratory. In the present study, the effect of antibodies on the susceptibility of HCV to the inactivation procedure was for the first time examined. The data suggest that HCV antibodies do not influence the sensitivity of viruses, indicating that MB/light treatment is equally efficient in units from seropositive or seronegative donors.

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